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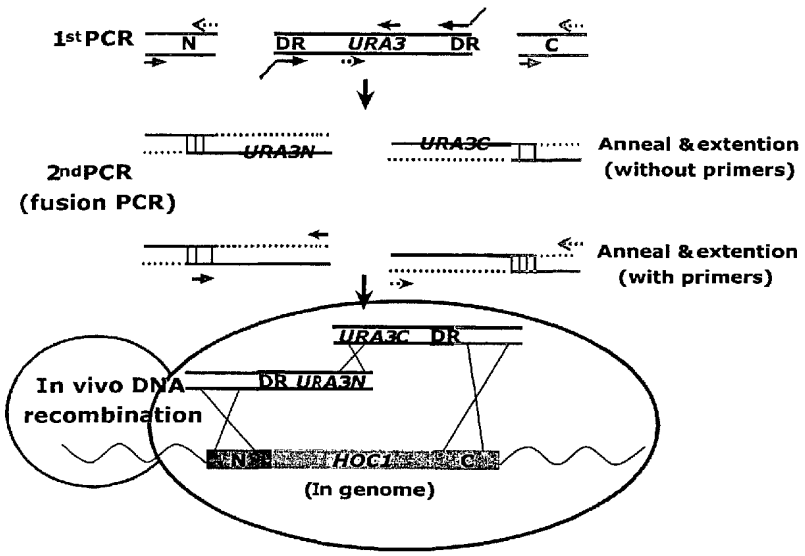
(54) Title: HANSENULA POLYMORPHA MUTANT STRAINS WITH DEFECT IN OUTER CHAIN BIOSYNTHESIS AND THE PRODUCTION OF RECOMBINANT GLYCOPROTEINS USING THE SAME STRAINS



(57) Abstract: The present invention relates to polynucleotide containing *Hansunula polymorpha Hpoch 1* gene; polypeptide coded thereby; *Hansunula polymorpha* mutant wherein hyperglycosylation of glycoprotein id inhibited by the mutation of the *Hansunula polymorpha HpOCH1*, or *Hansunula polymorpha* natural mutant; recombinant *Hansunula polymorpha* strain expressing a foreign protein prepared by introducing a gene coding a foreign protein to the *Hansunula polymorpha* mutant or *Hansunula polymorpha* natural mutant; and a method for preparing a foreign protein comprising the steps of culturing said mutant under the condition that a foreign protein can be expressed, and isolating the foreign protein from the obtained culture broth.

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For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

Hansenula polymorpha mutant strains with defect in outer chain biosynthesis and the production of recombinant glycoproteins using the same strains

Field of the invention

The present invention relates to *Hansenula polymorpha* mutant strains with a defect in the outer chain biosynthesis of glycoproteins and the production method of recombinant glycoproteins using these strains. More specifically, the present invention relates to the nucleic acid molecules containing *H. polymorpha* *HpOCH1* gene, the polypeptides encoded by it, and *H. polymorpha* artificial mutant strains or its natural mutant strains in which hyperglycosylation of glycoproteins is prevented. Furthermore, the present invention relates to recombinant *H. polymorpha* strains expressing a foreign protein produced by transformation with a gene encoding a foreign protein, and the production method of a foreign protein, which comprises cultivating the strains under conditions that allow them to express the foreign protein and isolating the expressed foreign protein from the cultures.

Background of the invention

In order to express a foreign protein recombinantly on a large scale, an optimal expression system should be selected to establish an efficient production system because amounts, solubility, locations and modifications etc. of expressed

proteins are dependent on host cell lines or features of desired proteins. For large-scale expression of proteins, various host systems including bacteria, yeasts, fungi, plants and animals have been developed. Among them, microbes have been wildly used to express recombinant proteins because of easy culture thus getting a high concentration of recombinant protein with a low-cost.

Yeasts, microbes having features of the eukaryotic expression and secretion of proteins, are a suitable expression system to produce recombinant proteins of higher eukaryotes on a large-scale. In comparison to bacterial expression systems, yeast expression systems have a major advantage in that, as eukaryotic microbes, they have protein secretory organelles similar to those of higher eukaryotes. Therefore, the secretory proteins in yeast become biologically active through post-translational modifications such as digestion of secretory signal sequences, formation of disulfide bonds, glycosylation etc. Furthermore, the expressed recombinant proteins can be easily recovered and purified, since most yeast cells secrete only a small fraction of the proteins to the outside.

Recently, methylotrophic yeasts such as *Hansenula polymorpha, Pichia pastoris* and other non-conventional yeasts have been developed as alternative hosts, because they are able to replace the inherent disadvantages of the traditioanl yeast *Saccharomyces cerevisiae* as hosts for industrial production of desired proteins. The disadvantages of *S. cerevisiae* include instability of expression vectors in long-term fermentation, hyperglycosylation of glycoproteins, and low productivity of the expressed proteins in comparison to

bacterial expression system (Gellissen, *Appl. Microbiol. Biotechnol.* 54, 741, (2000)).

Most proteins utilized for medical therapeutic purposes in humans are glycoproteins, which are modified by attachment of oligosaccharides via covalent bonds in a secretory pathway. An important issue in large-scale protein production in the field of biotechnology is the production of recombinant proteins modified by suitable glycosylation because the structures and classes of carbohydrates attached to the glycoproteins can greatly affect folding, secretion, stability, half-life in serum, and antibody inductivity of the proteins.

Wild type yeasts have some limits as an expression system. The recombinant glycoproteins expressed in *S. cerevisiae* have showed hypermannosylation resulting from adding over 40 mannose residues to the proteins and α 1,3-linked terminal mannose, which serves as an antigen in the human body (Romanos *et al.*, *Yeast* 8, 423-488, 1992). In contrast the recombinant proteins expressed in methylotrophic yeasts, *H. polymorpha* and *P. pastoris*, have been reported to contain the mannose outer chains that are shorter than those expressed in *S. cerevisiae* although they are still more hyperglycosylated than native proteins (Bretthauer and Castellino, *Biotechnol. Appl. Biochem.* 30, 193-200, 1999; Kang *et al.*, *Yeast* 14, 371-381, 1998). These methylotrophic yeasts are preferred over the wild type *S. cerevisiae* as a host system for medical therapeutic proteins because they do not produce the α 1,3-linked terminal mannose, which can evoke an immune response.

The core oligosaccharide is an intermediate of the biosynthesis pathway, which is found in all eukaryotes from yeasts to mammalian cells. However, the outer chains attached to the intermediate are differentially biosynthesized based on species of proteins, cells and animals. Researchers have actively pursued the development of a useful host system to produce recombinant glycoproteins, which closely resemble native proteins containing proper outer chains, by means of selecting mutant strains with defects in outer chain biosynthesis using an artificial mutant method or manipulating the gene related to the chain biosynthesis using molecular biological techniques.

In wild type *S. cerevisiae*, several strategies such as [^{3}H]mannose suicide selection, sodium orthovanadate resistance and hygromycin B sensitivity are used to select the defective mutants of N-linked oligosaccharides biosynthesis (Herscovics and Orlean, *FASEB* 7, 540-550, 1999). Functional complementation experiments using these mutants led to the cloning of the *OCH1* gene (*Ngd29*) playing an important role in the outer chain initiation (Nakanishi-Shindo *et al.*, *J. Biol. Chem.* 268, 26338-26345, 1993), the *MNN9* gene regulating the outer chain elongation and the *MNN1* gene involved in attachment of the α 1,3-linked terminal mannose (Gopal and Ballou, *Proc. Natl. Acad. Sci. USA* 84, 8824, 1987). Those genes were targeted to make defective mutants by mutagenesis, which were then developed as a host cell to produce recombinant glycoproteins (Kniskern *et al.*, *Vaccine* 12, 1021-1025, 1994; US Patent no. 5,798,226; US Patent no. 5,135,854).

Methylotrophic yeasts have recently been in the spotlight as a suitable host for recombinant protein expression over *S. cerevisiae*. However, a defective mutant of the N-linked oligosaccharide biosynthesis in methylotrophic yeasts has not yet been reported.

The goal of this invention was to develop a mutant using *H. polymorpha,* a methylotrophic yeast, which can produce recombinant glycoproteins that are suitable for use in the human body. This mutant was obtained by selection of a defective mutant in the glycosylation pathway or by mutation of the *OCH1* gene involved in the process. This defective mutant prevents hyperglycosylation of the outer chains and is a suitable host for recombinant glycoproteins attached with proper outer chains, which closely resemble the native proteins.

Summary of the invention

In order to develop a defective mutant of *H. polymorpha* for production of recombinant N-linked glycoproteins closely resembling those of human, we developed a method for selection of a defective mutant of the oligosaccharide chain biosynthesis. We used sensitivity of sodium orthovanadate to select a defective mutant of *H. polymorpha*, which exhibits more resistance against it. We also cloned the *OCH1* gene involved in initiation of the outer chain biosynthesis. The gene was mutated to make an *OCH1* deletion mutant (*Δoch1*) strain. This mutant strain is a suitable host, which provides techniques to produce

recombinant glycoproteins close to the structure of original proteins with proper outer oligosaccharide chains.

Brief description of the drawings

Figure 1 shows the difference in the resistance of *H. polymorpha* strains, against sodium orthovanadate.

Figure 2 shows the phenotype of the *H. polymorpha* mutant, DL42-15. Yeast cells in a log phase were serially diluted 1 to 10, 5 μl was spotted onto the YPD plate and the cells were cultured for 2 days. A, YPD media containing 4 mM sodium orthovanadate; B, YDP media at 45℃; C, YPD media containing 0.3% sodium deoxycholate; D, YDP media at 37℃.

Figure 3 shows the sequences of DNA and predicted amino acid of *H. polymorpha OCH1* gene cloned in this study.

Figure 4 shows amino acid sequence alignment of the Och1p of *H. polymorpha* with homologues of other yeast strains. The numbers in parentheses represent homology of Och1p from other yeast strains versus Och1p of *H. polymorpha*. HpOch1p; *H. polymorpha Och1* protein; ScOch1p, *S. cerevisiae Och1* protein; ScHoc1p, *S. cerevisiae Hoc1* protein; CaOch1p, *C. albicans Och1* protein.

Figure 5 is an illustration showing the gene recombination and pop-out to induce the *H. polymorpha OCH1* gene disruption

Figure 6 shows the phenotype of the *och1* defective mutant (*Δoch1*) of *H. polymorpha*. Yeast cells in a log phase were serially diluted 1 to 10, 5 μl was spotted onto the YPD plate and the cells were cultured for 2 days. A, YDP media at 37 ℃ ; B, YDP media at 45 ℃ ; C, YPD media containing 40 μg/ml of hygromycin B; D, YPD media containing 0.4% of sodium deoxycholate; E, YPD media containing 7 mg/ml of calcofluor white.

Figure 7 is a Western blot demonstrating the changes in the oligosaccharide formation of glucose oxidase expressed in the *H. polymorpha* mutant, DL42-15, and the *och1* defective strain (*Δoch1*).

Description of the preferred embodiment

The present invention consists of selecting the naturally occurring sodium vanadate-resistant mutant strain, DL42-15, originated from *H. polymorpha* DL-1 ; cloning the *H. polymorpha OCH1* gene and analyzing the DNA sequence; disrupting the *H. polymorpha OCH1* gene; testing for the glycosylation of the *Aspergillus niger* glucose oxidase protein expressed in the sodium orthovanadate-resistant strain, DL42-15, and the defective mutant strain, *Δoch1*.

The invention describes engineering of the defective mutant, which was mutated in the outer chain biosynthesis of a methylotrophic yeast *H. polymorpha* to prevent hyperglycosylation by subsequent attachment of mannose residues. This mutant is an ideal host for expression of human recombinant proteins because it produces glycoproteins with fewer outer chains that more closely resemble the native proteins and therefore do not initiate an immune response. The hyperglycosylation-inhibiting mutants originated from *H. polymorpha* DL-1 were either a natural mutant selected by sodium orthovanadate or the mutant mutated in the *OCH1* gene of *H. polymorpha*.

The DNA sequence (nucleotide no. 1) of *H. polymorpha OCH1* cloned in this study was deposited in GenBank (accession no. AF490971) and in the Korean Collection for Type Culture (KCTC) on May 29, 2002 (accession no. KCTC 10265BP). The sodium orthovanadate-resistant strain, DL42-15, and the *OCH1* gene-mutated strain, *Δoch1*, of *H. polymorpha* were also deposited in the KCTC on the same day (accession no. KCTC 10263BP and KCTC 10264BP, respectively).

This invention provides the DNA and amino acid sequences shown in Figure 3.

This invention provides the *OCH1* gene mutant (*Δoch1*), which inhibits hyperglycosylation of glycoproteins.

This invention provides this mutant yeast strain as an expression host to express genes encoding heterologous glycoproteins.

This invention provides the hyperglycosylation-inhibiting mutant yeast strain, DL42-15, deposited in KCTC (accession no. KCTC 10263 BP).

This invention provides this DL42-15 strain as an expression host to express genes encoding heterologous glycoproteins.

This invention provides suitable conditions for cell culture of these mutants as well as methods for the production and isolation of the recombinant proteins from the culture.

Methylotrophic yeasts such as *H. polymorpha* and *P. pastoris* have been extensively used for production of therapeutic recombinant proteins in medical and pharmaceutical industries.

The term "hyperglycosylation-inhibiting" used in this study refers to reduction of the oligosaccharide chains attached to glycoproteins expressed in the mutants of the methylotrophic yeasts in comparison of those of the wild-type yeasts.

The term "glycoproteins" used in this study refers to proteins processing glycosylation on more than one residue of asparagine, serine or threonine of glycoproteins in *H. polymorpha*.

Possible glycoproteins that can be produced using these invented mutants include, but are not limited to, the *Aspergillus niger* glucose oxidase, the *S. cerevisiae* invertase, the HIV envelop protein, the influenza A virus hemagglutinin, the influenza neuraminidase, the bovine herpes type-1 virus glycoprotein D, the human angiostatin, erythropoietin, cytokine, human B7-1, B7-2, B-7 receptor CTLA-4, human tissue factors, human growth factors (e.g. blood

platelet-derived growth factor), tissue plasminogen activator, plasminogen activator inhibitor-1, eurokinase, human lysosomal enzymes (e.g. α-galactosidase), plasminogen, thrombin, factor XIII and immune globulin. Those glycoproteins can be used for therapeutic medicine delivered by injection, oral or non-oral administration or other methods used in particular areas.

Glycoproteins produced in the mutants can be isolated and purified using general methods for protein isolation and purification. However, the specific methods employed depend on the property of the proteins to be isolated. These properties should be determined by the parties interested. In brief, cultured cells are collected, the secreted proteins are precipitated, and the proteins are isolated and purified according to a general method for protein isolation and purification using immune absorption, fractionation or chromatography

The following examples explain the invention in detail, however, the claims are not limited to them.

<Experimental example 1>

Selection of the sodium orthovanadate-resistant mutant strain, DL42-15, of *H. polymorpha*

Even a low concentration (5 mM) of sodium orthovanadate generally inhibits the growth of yeast. Most *S. cerevisiae* vanadate-resistantmutant strainsare mutants with mutations in genes involved in glycosylation processing

in the Golgi (Kanik-Ennulat *et al.*, *Genetics* 140; 933-943, 1995); Uccelletti *et al.* *Res Microbiol* 150:5-12, 1999). One of the most efficient methods for selection for oligosaccharide biosynthesis defective mutants is using sodium orthovanadate to select one with its resistance and this method has been extensively used in *S. cerevisiae* and *Kluyveromyces lactis*. However, this method cannot be used in the methylotrophic yeast *P. pastoris* because it itself is resistant to sodium orthovanadate (Martinet *et al.*, *Biotechnology Lett.* 20, 1171-1177, 1999). In the case of another methylotrophic yeast *H. polymorpha,* CBS 4732 and NCYC 495 strains have also been reported that they can grow in the media containing 96 mM sodium orthovanadate (Mannazzu *et al.*, *FEMS Microbiol Lett.* 147: 23-28, 1997; Mannazzu *et al. Microbiology* 144: 2589-2597, 1998).

The *H. polymorpha* DL1, used in this study to develop a expression host for production of recombinant proteins, showed a similar sensitivity to sodium orthovanadate to *S. cerevisiae* unlike CBS 4732 and NCYC 495 (Figure 1 and Table 1). The natural mutant cells of *H. polymorpha* DL1, which became resistant to the sodium orthovanadate , occurred at a frequency of 1 per10^6 cells on the YPD media plate containing 4 mM sodium orthovanadate. showed that This mutation frequency is similar to that in the wild type *S. cerevisiae* (Table 1).

[Table 1]

Growth comparison of yeast strains grown on the YPD plates containing sodium orthovanadate.

Yeast strains	Sodium orthovanadate (mM)				
	4	6	8	10	12
S. cerevisiae L3262 (WT)	±	-	-	-	-
S. cerevisiae L3262 (*mnn9*)	++++	+++	+	-	-
H. polymorpha DL1 (WT)	±	-	-	-	-
H. polymorpha CBS4732 (WT)	+++++	+++++	+++++	++++	+++

* The results were obtained after culturing at 30℃ (*S. cerevisiae*)or at 37℃ (*H. polymorpha*) for 4 days.

All the defective mutants of oligosaccharide biosynthesis among the sodium orthovanadate-resistant mutants of *S. cerevisiae* have been shown to be more sensitive to antibiotics with a large molecular weight such as aminoglycoside, to synthetic detergents such as sodium deoxycholate, and to high temperature (Dean N., *Proc. Natl. Acad. Sci. USA* 92, 1287-1291, 1995). We selected 250 natural mutants from *H. polymorpha* DL1 showing more resistance to sodium orthovanadate, most (over 90%) of which were also resistant to hygromycin B. The selected mutants have been further tested on the media containing sodium orthovanadate at high temperature (45℃) to select the mutant colonies resistant to sodium orthovanadate but sensitive to high temperature. Finally, the mutants have been isolated and designated as *H. polymorpha* DL42-15 (Figure 2 and 3).

<Experimental example 2>

Cloning and DNA sequence analysis of the *H. polymorpha OCH1* gene

We analyzed the Random Sequenced Tags (RSTs) of the partial genomic analysis of *H. polymorpha* (Blandin *et al.*, *FEBS Lett.* 487, 76, 2000) and obtained the partial DNA sequences of genes showing homology with the genes involved in the oligosaccharide biosynthesis of *S. cerevisiae*. The predicted amino acid sequences deduced from the partial DNA sequences share homology with a region corresponding to the C-termini of *S. cerevisiae OCH1* (*ScOCH1*), which plays an important role in attachment of α1, 6-mannose in the beginning of the outer chain biosynthesis. *S. cerevisiae ScOCH1* also shares high homology to *S. cerevisiae HOC1* (*ScHOC1*). A pair of primers designed based on the partial DNA sequences are 5'-CAATCAGACCCGGTCTGTCGAGGAGT-3'(nucleotide no. 3), 5'-ACATCAACGTGGAGAACTGGGAGCAC-3' (nucleotide no. 4). Using these primers, we amplified by PCR a 900 bp fragment from genomic DNA isolated from *H. polymorpha*.

We performed Southern blotting, probed with the 900 bp fragment, using the genomic DNAs digested with several restriction enzymes. In order to isolate the promoter region and full-length of the *H. polymorpha OCH1* gene, we gel-extracted the two fragments of 2.3 kb (digested with *BamHI*) and 5 kb (digested with *BglII*) corresponding to the signals of the Southern blot. Each fragment was then cloned into a cloning vector pBluescript KS+ (Stratagen Co.). The clones were sequenced in both strands.

The DNA sequence analysis revealed the clones include the promoter region of 1 kb and the open reading frame of 1.3 kb encoding a putative protein

with 435 amino acids (nucleotide no. 1, Figure 3). The predicted protein of *H. polymorpha* was designated as *HpOch1* (amino acid sequence no.2). This protein shares low homology (21-23%) to *ScOCH1* (accession no. YGL038C), *ScHOC1* (accession no. YJR075W) and *Candida albicans Och1* (accession no. AY064420) *proteins*. However, it contains a DXD motif, a possible activation site, and the transmembrane spanning region in the N-terminal found in the mannosyltransferase, a type II membrane protein (Figure 4).

<Experimental example 3>

Production and analysis of the *OCH1* gene-mutated strain (Δ *och1*) of *H. polymorpha*

In order to make the mutants where the *OCH1* gene was disrupted, two techniques, fusion PCR using the primers listed in Table 2 and *in vivo* DNA recombination, were used for the gene disruption (Oldenburg *et al.*, *Nucleic Acid Res.* 25, 451, 1997). The regions corresponding to the N-terminal and the C-terminal of *URA3* and *OCH1* genes, respectively, were amplified by PCR. The fragment corresponding to the N-terminal of *HpOCH1* was then fused by fusion PCR to the fragment corresponding to the N-terminal of *URA3* while the fragment corresponding to the C-terminal of *HpOCH1* was fused to the fragment corresponding to the C-terminal of *URA3*. The fused DNA fragments were introduced into yeast cells to make recombination of the gene. Transformants

where the *HpOCH1* gene was disrupted were then selected (Figure 5). The mutants were first screened on the minimal media containing no uracil, selecting for the *URA3* marker. PCR was then performed on the genomic DNAs isolated from the mutants and the wild type to confirm the *HpOCH1* gene disruption. An *H. polymorpha* mutant *Δoch1*(*leu2 och1::URA3*) was selected based on analysis of the PCR products.

The selected mutant strain *Δoch1* grows more slowly than the wild type; it is more sensitive to a high temperature of 45℃ and to hygromysin B; its growth is inhibited by addition of sodium orthovanadate and calcofluor white (Figure 6). All these properties are common in the defective mutant strains of the outer chain biosynthesis in yeasts, suggesting the mutant strain *Δoch1* has a defect in the biosynthesis.

[Table 2]

Primers used in this study for PCR to disrupt the *HpDCH1* gene

Name	Oligonucleotide sequences (5' to 3')	Nucleotide no.
OCH1 N-S	ACATCAACGTGGAGAACTGG	5
OCH1 N-A	AGCTCGGTACCCGGGGATCCTGTCTGTCCACAC AACAGG	6
OCH1 C-S	GCACATCCCCCTTTCGCCAGCCCATACACTCCTT ACTAGG	7
OCH1 C-A	CAATCAGACCCGGTCTGTCGAGGAGT	8
URA3 N-S	GGATCCCCGGGTACCGAGCT	9
URA3 N-A	CACCGGTAGCTAATGATCCC	10
URA3	CGAACATCCAAGTGGGCCGA	11

C-S		
URA3 C-A	CTGGCGAAAGGGGGATGTGC	12

<Experimental example 4>

Analysis of the recombinant glycoproteins expressed and isolated from the mutant strains, DL42-15 and *Δoch1*, of *H. polymorpha*.

In order to examine the glycosylation defect on a recombinant glycoprotein expressed in the mutant strains, DL42-15 and *Δoch1*, described in experimental example 1 and 3 respectively, we expressed the glucose oxidase (GOD) of an *Aspergillus niger* gycoprotein in these mutants. The GOD protein contains the 8 potential sites for the N-linked glycosylation (Frederick *et al.*, *J. Biol. Chem.* 265, 3793, 1990).

In order to express the GOD in the mutant yeast strains, we constructed a GOD expression vector, pDLMOX-GOD using the pDLMOX-Hir vector (Kang *et al. Yeast* 14, 371, 1998)). The DNA fragment containing the hirudin gene was first removed from the pDLMOX-Hir vector and the GOD gene fused to the fragment corresponding to the secretory signal of the α-amylase at the N-terminal was then replaced in the vector (Kim S. Y. Ph. D. Dissertations, Yonsei University, Korea, 2001). The resultant vector pDLMOX-GOD was introduced into the two mutant strains, DL42-15 and *Δoch1* as well as the wild type strain,

and they were cultured on the YPM media (1% yeast extract, 2% peptone, 2% methanol) to express the GOD proteins.

The GOD proteins expressed and secreted were isolated and purified for Western blot anlaysis. The proteins were run on a polyarcylamide gel, transferred to a nitrocellulose membrane, and blotted using a GOD antibody. Figure 7A shows that the GOD proteins of the mutant strains, DL42-15 and *Δoch1*, have a smaller molecular weight than that of the wild type, suggesting the proteins expressed and secreted in the mutants are less hyperglycosylated, or in other words, hyperglycosylation is inhibited in the mutant strains. To confirm the blotting result, we treated all the proteins with endoglycosidase H enzyme to digest the oligosaccharide chains attached on the proteins, and repeated the blot. Figure 7B shows that all the proteins have the same molecular weight on the blot, suggesting they are all the same proteins. These results demonstrate that the proteins expressed and secreted in the mutant cells were smaller than the one expressed and secreted in wild type cells due to less hyperglycosylation on the proteins. Therefore, the mutant strains, DL42-15 and *Δoch1*, unlike the wild type, are suitable host cells to produce the human glycoproteins, in which the hypergylcosylation of the proteins will be inhibited, resulting in a closer resemblance to native human proteins.

Possible application of the invention to industries

The *H. polymorpha* mutants, DL42-15 and *Δoch1*, are able to be used as host cells to produce recombinant glycoproteins, which will express and secrete the proteins containing proper outer oligosaccharide chains closely resembling the native proteins because the hyperglycosylation of the proteins is inhibited in the mutants cells. These mutants will be useful in the medical therapeutic industry because *H. polymorpha* yeast cells has been broadly used to produce medical therapeutic recombinant proteins on a large scale.

Applicant's or agent's	International application No.
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29/05/2002	KCTC 10264BP

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D.DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE *(if the indications are not for all designated States)*

E.SEPARATE FURNISHING OF INDICATIONS *(leave blank if not applicable)*

The indications listed below will be submitted to the International Bureau later *(specify the general nature of the indications e.g., "Accession Number of Deposit")*

For receiving Office use only	For international Bureau use only
☐ This sheet was received with the international application	☐ This sheet was received by the International Bureau on:
Authorized officer	Authorized officer

What Is Claimed Is:

1. A nucleic acid molecule comprising the DNA sequence shown in Figure 3.

2. The nucleic acid molecule according to claim 1, wherein the nucleic acid molecule is *Hansenula polymorpha HpOCH1* gene (KCTC 10265BP).

3. A polypeptide comprising the amino acid sequence shown in Figure 3.

4. *H. polymorpha* mutant strains which prevent the hyperglycosylation of glycoproteins by mutation of the *HpOCH1* gene.

5. A *H. polymorpha* mutant strain *Δoch1* (KCTC 10264BP) according to claim 4, wherein the *HpOCH1* gene is disrupted. .

6. A recombinant *H. polymorpha* strain expressing a foreign protein, wherein the recombinant strain is produced by introducing the gene encoding the foreign protein into *H. polymorpha* strain according to claim 4.

7. A recombinant *H. polymorpha* strain according to claim 6 expressing a foreign protein, wherein the recombinant strain is produced by introducing the gene encoding the foreign protein into the disrupted mutant strain, *Δoch1* .

8. A natural mutant strain, DL-42-15, originated from *H. polymorpha* DL1 strain, prevented from hyperglycosylation of glycoproteins (KCTC 10263BP).

9. A recombinant *H. polymorpha* strain expressing a foreign protein, wherein the recombinant strain is produced by introducing the gene encoding the foreign protein into *H. polymorpha* mutant strain, DL-42-15 according to claim 8

10. A method for producing a foreign protein, wherein the method comprises cultivating the recombinant strains according to any one of claims 6, 7, and 9 under conditions that allow the strains to express the foreign protein and isolating the expressed foreign protein from the cultures.

11. The method according to claim 10, wherein hyperglycosylation of a foreign protein is prevented.

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FIG. 1

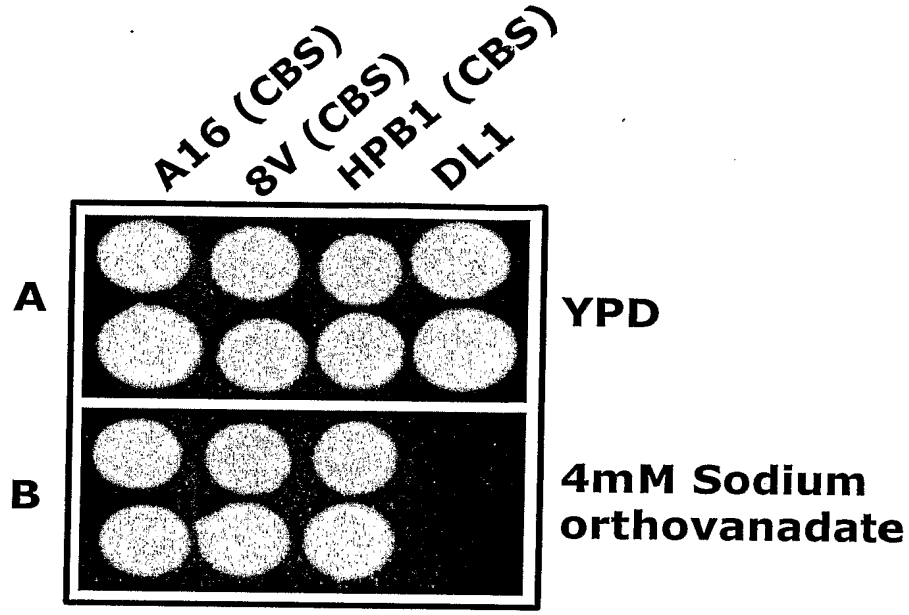
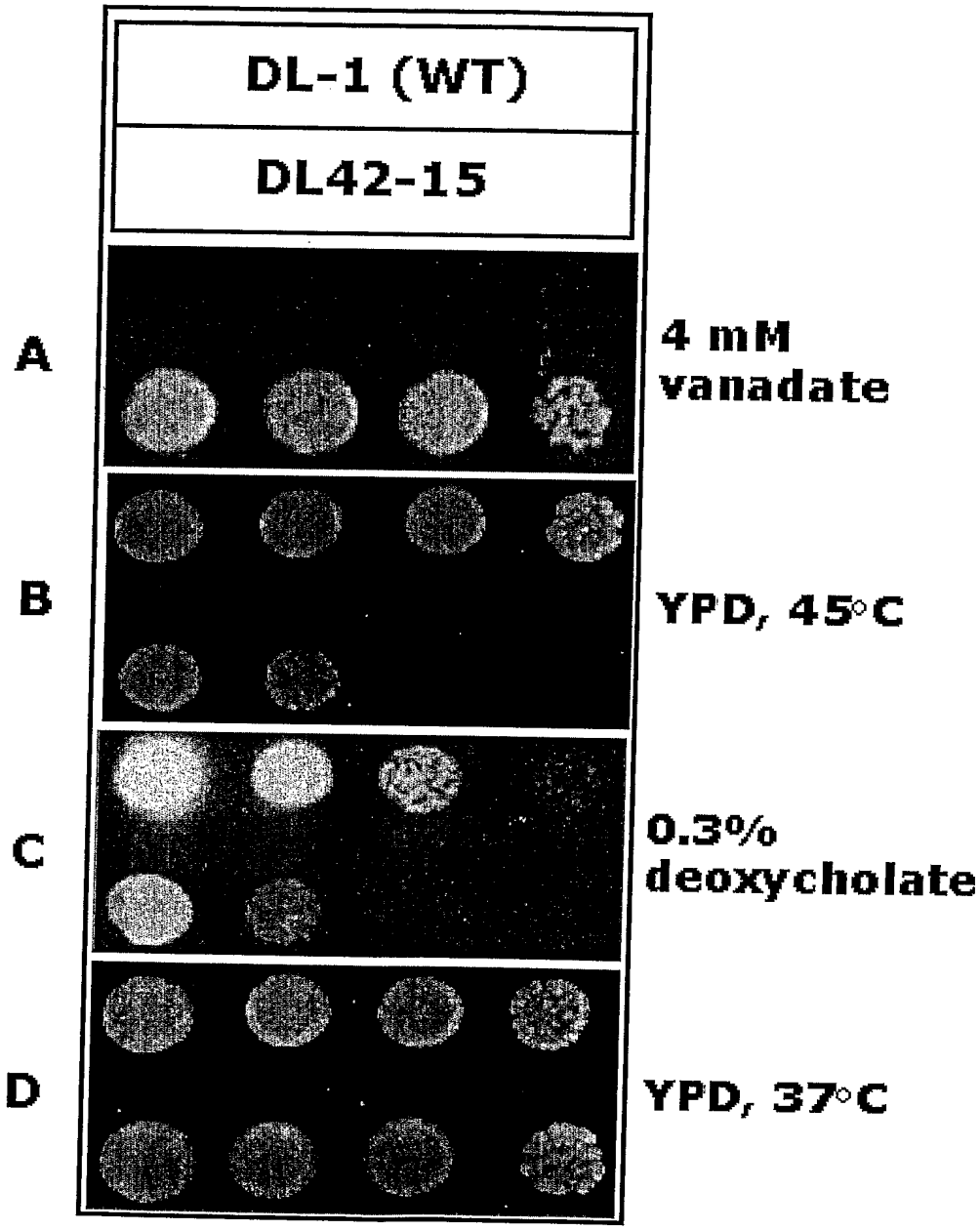


FIG. 2


DL-1 (WT)
DL42-15
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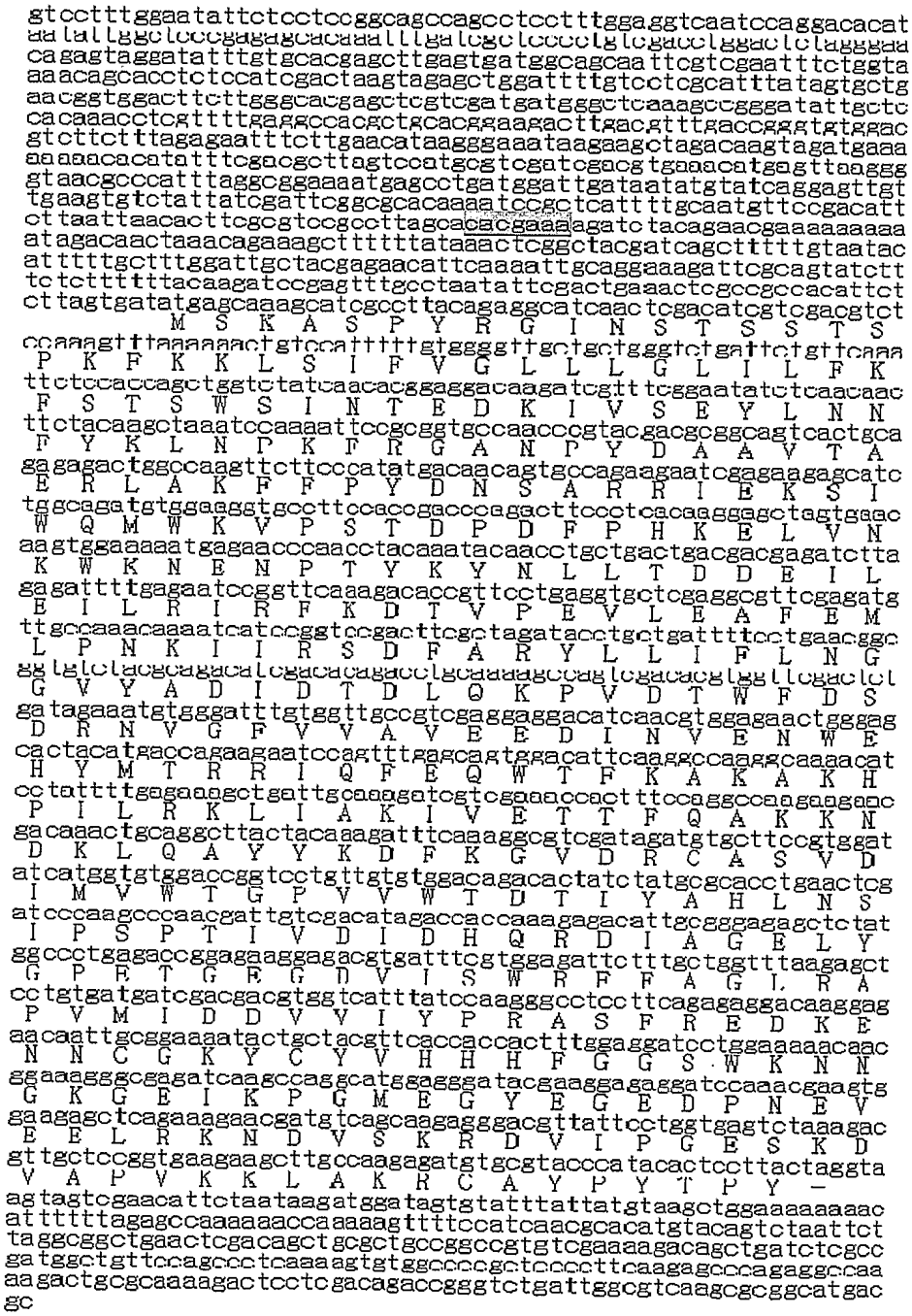
B
YPD, 45°C
C
0.3% deoxycholate
D
YPD, 37°C

FIG. 3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ctaagtgatatgagcaaagcatcgccttacagaggcatcaactcgacatcgtcgacgtct
M S K A S P Y R G I N S T S S T S
P K F K K L S I F V G L L L G L I L F K
F S T S W S I N T E D K I V S E Y L N N
F Y K L N P K F R G A N P Y D A A V T A
E R L A K F F P Y D N S A R R I E K S I
W Q M W K V P S T D P D F P H K E L V N
K W K N E N P T Y K V N L L T D D E I L
E I L R I R F K D T V P E V L E A F E M
L P N K I I R S D F A R Y L L I F L N G
G V Y A D I D T D L Q K P V D T W F D S
D R N V G F V V A V E E D I N V E N W E
H Y M T R R I Q F E Q W T F K A K A K H
P I L R K L I A K I V E T T F Q A K K N
D K L Q A Y Y K D F K G V D R C A S V D
I M V W T G P V V W T D T I Y A H L N S
I P S P T I V D I D H Q R D I A G E L Y
G P R T G E G D V I S W R F F A G L R A
P V M I D D V V I Y P R A S F R E D K E
N N C G K Y C Y V H H H F G G S W K N N
G K G E I K P G M E G Y E G E D P N E V
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V A P V K K L A K R C A Y P Y T P Y –

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FIG. 4

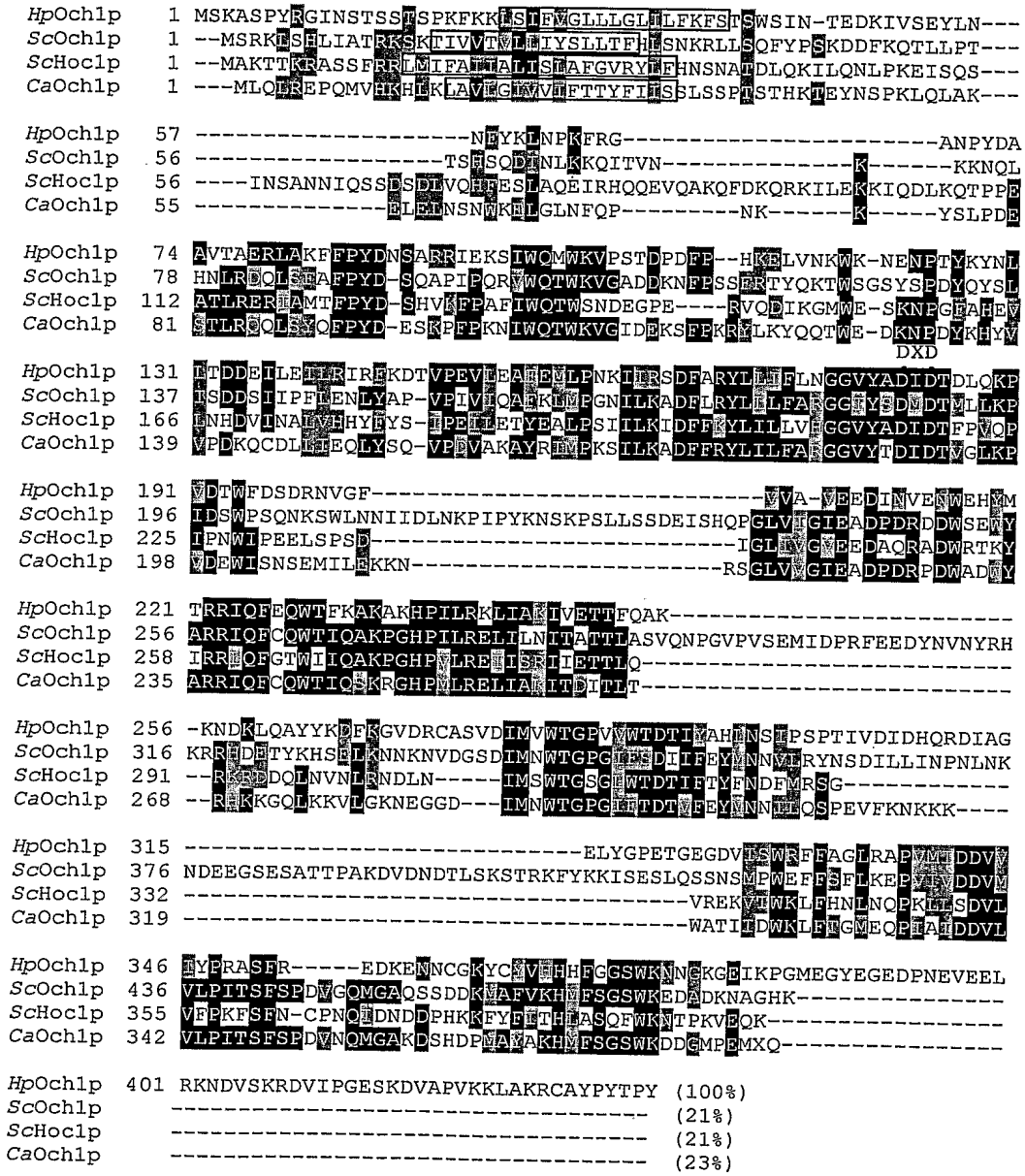

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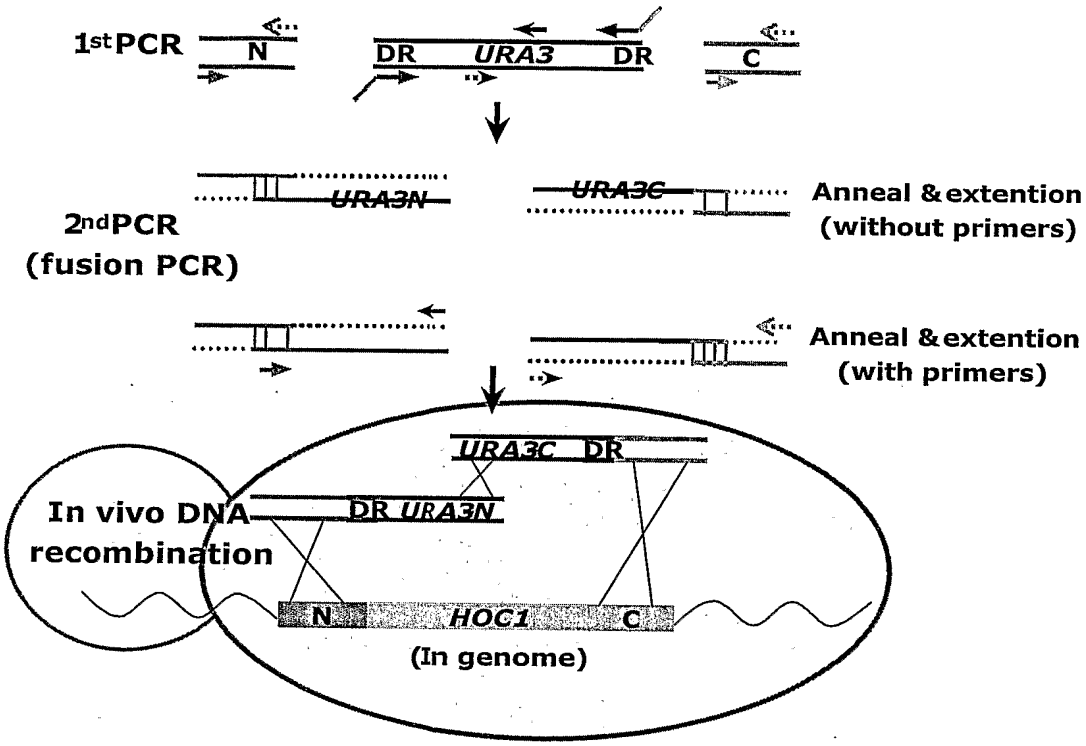
FIG. 5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N
DR
URA3
DR
C
URA3N
URA3C
Anneal & extention
(without primers)
2nd PCR
(fusion PCR)
Anneal & extention
(with primers)
URA3C
DR
In vivo DNA
recombination
DR URA3N
N
HOC1
C
(In genome)

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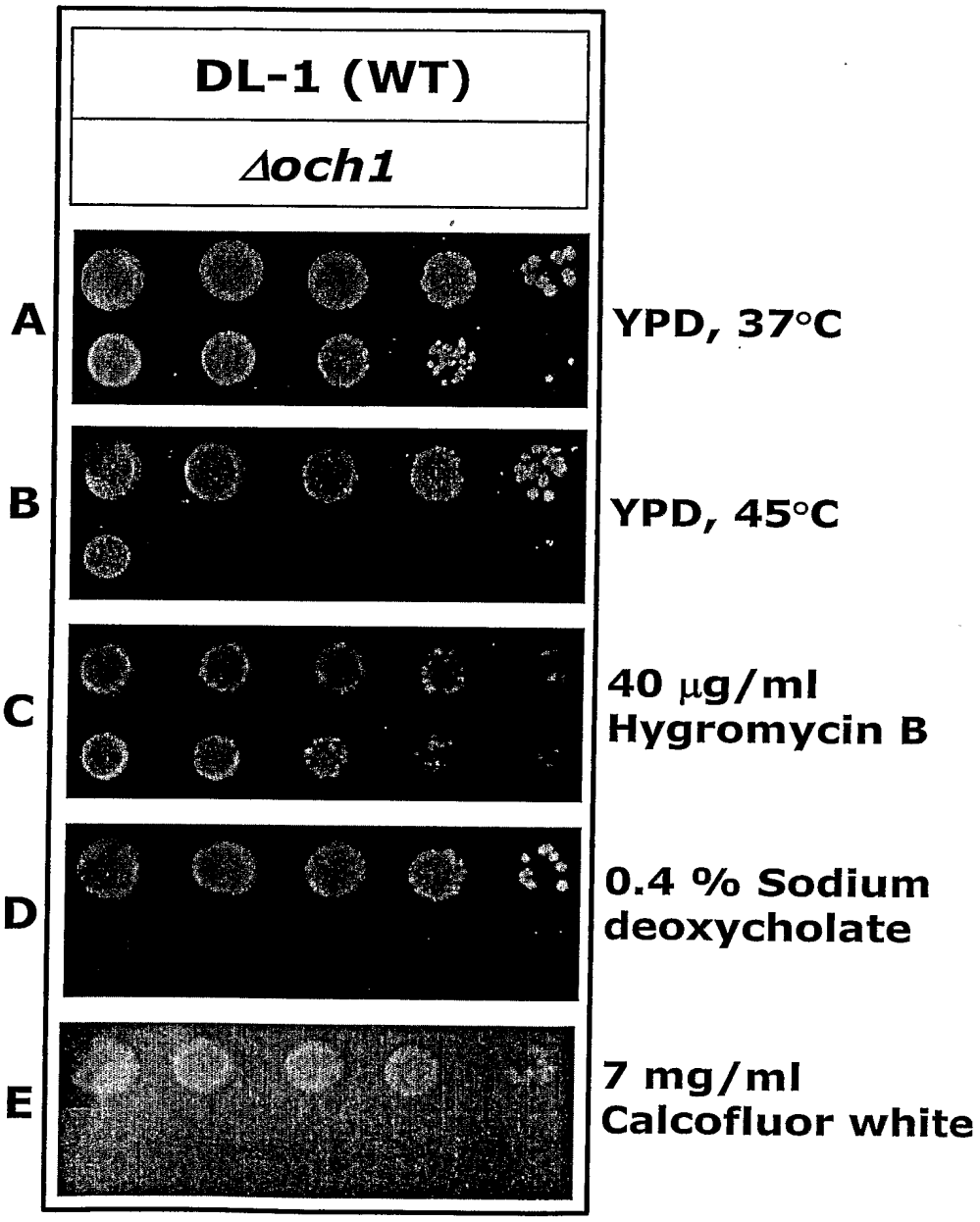
FIG. 6


DL-1 (WT)
Δoch1
A
YPD, 37°C
B
YPD, 45°C
C
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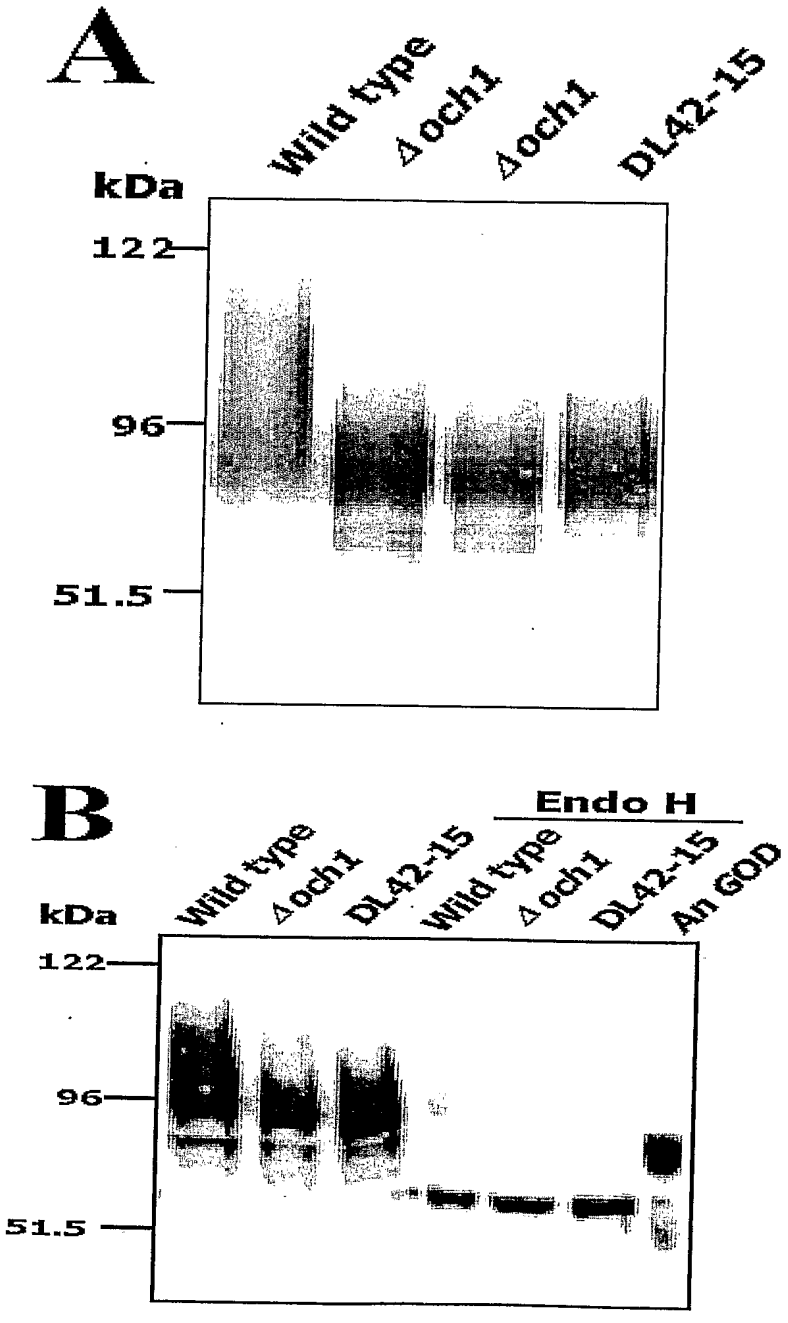
Hygromycin B
D
0.4 % Sodium
deoxycholate
E
7 mg/ml
Calcofluor white

FIG. 7


A
Wild type
Δoch1
Δoch1
DL42-15
kDa
122
96
51.5
B
Endo H
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Δoch1
DL42-15
Wild type
Δoch1
DL42-15
An GOD
kDa
122
96
51.5

Sequence Listing

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<120> Hansenula polymorpha mutant strains with defect in outer chain biosynthesis and the production of recombinant glycoproteins using the same strains

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<222> (1)..(849)

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<222> (2158)..(2462)

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cagagtagga tatttgtgca cgagcttgag tgatggcagc aattcgtcga atttctggta 180

aaacagcacc tctccatcga ctaagtagag ctggattttg tcctcgcatt tatagtgctg 240

aacggtggac ttcttgggca cgagctcgtc gatgatgggc tcaaagccgg gatattgctc 300

cacaaacctc gttttgaggc cacgctgcac ggaagacttg acgtttgacc gggtgtggac 360

gtcttcttta gagaatttct tgaacataag ggaaataaga agctagacaa gtagatgaaa 420

aaaaacacat atttcgacgc ttagtccatg cgtcgatcga cgtgaaacat gagttaaggg 480

gtaacgccca tttaggcgga aaatgagcct gatggattga taatatgtat caggagttgt 540

tgaagtgtct attatcgatt cggcgcacaa aatccgctca ttttgcaatg ttccgacatt 600

cttaattaac acttcgcgtc cgccttagca cacgaaaaga tctacagaac gaaaaaaaaa 660

atagacaact aaacagaaag ctttttttata aactcggcta cgatcagctt tttgtaatac 720

atttttgctt tggattgcta cgagaacatt caaaattgca ggaaagattc gcagtatctt 780

tctctttttt acaagatccg agtttgccta atattcgact gaaactcgcc gccacattct 840

cttagtgat atg agc aaa gca tcg cct tac aga ggc atc aac tcg aca tcg 891
Met Ser Lys Ala Ser Pro Tyr Arg Gly Ile Asn Ser Thr Ser
1 5 10

tcg acg tct cca aag ttt aaa aaa ctg tcc att ttt gtg ggg ttg ctg 939
Ser Thr Ser Pro Lys Phe Lys Lys Leu Ser Ile Phe Val Gly Leu Leu
15 20 25 30

ctg ggt ctg att ctg ttc aaa ttc tcc acc agc tgg tct atc aac acg 987
Leu Gly Leu Ile Leu Phe Lys Phe Ser Thr Ser Trp Ser Ile Asn Thr

Sequence Listing

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gag gac aag atc gtt tcg gaa tat ctc aac aac ttc tac aag cta aat        1035
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            50                  55                  60

cca aaa ttc cgc ggt gcc aac ccg tac gac gcg gca gtc act gca gag        1083
Pro Lys Phe Arg Gly Ala Asn Pro Tyr Asp Ala Ala Val Thr Ala Glu
        65                  70                  75

aga ctg gcc aag ttc ttc cca tat gac aac agt gcc aga aga atc gag        1131
Arg Leu Ala Lys Phe Phe Pro Tyr Asp Asn Ser Ala Arg Arg Ile Glu
    80                  85                  90

aag agc atc tgg cag atg tgg aag gtg cct tcc acc gac cca gac ttc        1179
Lys Ser Ile Trp Gln Met Trp Lys Val Pro Ser Thr Asp Pro Asp Phe
95                  100                 105                 110

cct cac aag gag cta gtg aac aag tgg aaa aat gag aac cca acc tac        1227
Pro His Lys Glu Leu Val Asn Lys Trp Lys Asn Glu Asn Pro Thr Tyr
                115                 120                 125

aaa tac aac ctg ctg act gac gac gag atc tta gag att ttg aga atc        1275
Lys Tyr Asn Leu Leu Thr Asp Asp Glu Ile Leu Glu Ile Leu Arg Ile
            130                 135                 140

cgg ttc aaa gac acc gtt cct gag gtg ctc gag gcg ttc gag atg ttg        1323
Arg Phe Lys Asp Thr Val Pro Glu Val Leu Glu Ala Phe Glu Met Leu
        145                 150                 155

cca aac aaa atc atc cgg tcc gac ttc gct aga tac ctg ctg att ttc        1371
Pro Asn Lys Ile Ile Arg Ser Asp Phe Ala Arg Tyr Leu Leu Ile Phe
    160                 165                 170

ctg aac ggc ggt gtc tac gca gac atc gac aca gac ctg caa aag cca        1419
Leu Asn Gly Gly Val Tyr Ala Asp Ile Asp Thr Asp Leu Gln Lys Pro
175                 180                 185                 190

gtc gac acg tgg ttc gac tct gat aga aat gtg gga ttt gtg gtt gcc        1467
Val Asp Thr Trp Phe Asp Ser Asp Arg Asn Val Gly Phe Val Val Ala
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Sequence Listing

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                195                 200                 205

gtc gag gag gac atc aac gtg gag aac tgg gag cac tac atg acc aga        1515
Val Glu Glu Asp Ile Asn Val Glu Asn Trp Glu His Tyr Met Thr Arg
            210                 215                 220

aga atc cag ttt gag cag tgg aca ttc aag gcc aag gca aaa cat cct        1563
Arg Ile Gln Phe Glu Gln Trp Thr Phe Lys Ala Lys Ala Lys His Pro
        225                 230                 235

att ttg aga aag ctg att gca aag atc gtc gaa acc act ttc cag gcc        1611
Ile Leu Arg Lys Leu Ile Ala Lys Ile Val Glu Thr Thr Phe Gln Ala
    240                 245                 250

aag aag aac gac aaa ctg cag gct tac tac aaa gat ttc aaa ggc gtc        1659
Lys Lys Asn Asp Lys Leu Gln Ala Tyr Tyr Lys Asp Phe Lys Gly Val
255                 260                 265                 270

gat aga tgt gct tcc gtg gat atc atg gtg tgg acc ggt cct gtt gtg        1707
Asp Arg Cys Ala Ser Val Asp Ile Met Val Trp Thr Gly Pro Val Val
                275                 280                 285

tgg aca gac act atc tat gcg cac ctg aac tcg atc cca agc cca acg        1755
Trp Thr Asp Thr Ile Tyr Ala His Leu Asn Ser Ile Pro Ser Pro Thr
            290                 295                 300

att gtc gac ata gac cac caa aga gac att gcg gga gag ctc tat ggc        1803
Ile Val Asp Ile Asp His Gln Arg Asp Ile Ala Gly Glu Leu Tyr Gly
        305                 310                 315

cct gag acc gga gaa gga gac gtg att tcg tgg aga ttc ttt gct ggt        1851
Pro Glu Thr Gly Glu Gly Asp Val Ile Ser Trp Arg Phe Phe Ala Gly
    320                 325                 330

tta aga gct cct gtg atg atc gac gac gtg gtc att tat cca agg gcc        1899
Leu Arg Ala Pro Val Met Ile Asp Asp Val Val Ile Tyr Pro Arg Ala
335                 340                 345                 350

tcc ttc aga gag gac aag gag aac aat tgc gga aaa tac tgc tac gtt        1947
Ser Phe Arg Glu Asp Lys Glu Asn Asn Cys Gly Lys Tyr Cys Tyr Val
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Sequence Listing

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His His His Phe Gly Gly Ser Trp Lys Asn Asn Gly Lys Gly Glu Ile
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aag cca ggc atg gag gga tac gaa gga gag gat cca aac gaa gtg gaa        2043
Lys Pro Gly Met Glu Gly Tyr Glu Gly Glu Asp Pro Asn Glu Val Glu
        385                 390                 395

gag ctc aga aag aac gat gtc agc aag agg gac gtt att cct ggt gag        2091
Glu Leu Arg Lys Asn Asp Val Ser Lys Arg Asp Val Ile Pro Gly Glu
    400                 405                 410

tct aaa gac gtt gct ccg gtg aag aag ctt gcc aag aga tgt gcg tac        2139
Ser Lys Asp Val Ala Pro Val Lys Lys Leu Ala Lys Arg Cys Ala Tyr
415                 420                 425                 430

cca tac act cct tac       taggta agtagtcgaa cattctaata agatggatag    2190
Pro Tyr Thr Pro Tyr
                435

tgtatttatt atgtaagctg gaaaaaaaac atttttttaga gccaaaaaac caaaaagttt    2250

tccatcaacg cacatgtaca gtctaattct taggcggctg aactcgacag ctgcgctgcc    2310

ggccgtgtcg aaaagacagc tgatctcgcc gatggctgtt ccagccctca aaagtgtggc    2370

cccgctcccc ttcaagagcc cagaggccaa aagactgcgc aaaagactcc tcgacagacc    2430

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<213> Hansenula polymorpha

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Sequence Listing

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Leu Ile Leu Phe Lys Phe Ser Thr Ser Trp Ser Ile Asn Thr Glu Asp
        35                  40                  45

Lys Ile Val Ser Glu Tyr Leu Asn Asn Phe Tyr Lys Leu Asn Pro Lys
    50                  55                  60

Phe Arg Gly Ala Asn Pro Tyr Asp Ala Ala Val Thr Ala Glu Arg Leu
65                  70                  75                  80

Ala Lys Phe Phe Pro Tyr Asp Asn Ser Ala Arg Arg Ile Glu Lys Ser
                85                  90                  95

Ile Trp Gln Met Trp Lys Val Pro Ser Thr Asp Pro Asp Phe Pro His
            100                 105                 110

Lys Glu Leu Val Asn Lys Trp Lys Asn Glu Asn Pro Thr Tyr Lys Tyr
        115                 120                 125

Asn Leu Leu Thr Asp Asp Glu Ile Leu Glu Ile Leu Arg Ile Arg Phe
    130                 135                 140

Lys Asp Thr Val Pro Glu Val Leu Glu Ala Phe Glu Met Leu Pro Asn
145                 150                 155                 160

Lys Ile Ile Arg Ser Asp Phe Ala Arg Tyr Leu Leu Ile Phe Leu Asn
                165                 170                 175

Gly Gly Val Tyr Ala Asp Ile Asp Thr Asp Leu Gln Lys Pro Val Asp
            180                 185                 190

Thr Trp Phe Asp Ser Asp Arg Asn Val Gly Phe Val Val Ala Val Glu
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Glu Asp Ile Asn Val Glu Asn Trp Glu His Tyr Met Thr Arg Arg Ile
    210                 215                 220
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Sequence Listing

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Gln Phe Glu Gln Trp Thr Phe Lys Ala Lys Ala Lys His Pro Ile Leu
225                 230                 235                 240

Arg Lys Leu Ile Ala Lys Ile Val Glu Thr Thr Phe Gln Ala Lys Lys
                245                 250                 255

Asn Asp Lys Leu Gln Ala Tyr Tyr Lys Asp Phe Lys Gly Val Asp Arg
            260                 265                 270

Cys Ala Ser Val Asp Ile Met Val Trp Thr Gly Pro Val Val Trp Thr
        275                 280                 285

Asp Thr Ile Tyr Ala His Leu Asn Ser Ile Pro Ser Pro Thr Ile Val
    290                 295                 300

Asp Ile Asp His Gln Arg Asp Ile Ala Gly Glu Leu Tyr Gly Pro Glu
305                 310                 315                 320

Thr Gly Glu Gly Asp Val Ile Ser Trp Arg Phe Phe Ala Gly Leu Arg
                325                 330                 335

Ala Pro Val Met Ile Asp Asp Val Val Ile Tyr Pro Arg Ala Ser Phe
            340                 345                 350

Arg Glu Asp Lys Glu Asn Asn Cys Gly Lys Tyr Cys Tyr Val His His
        355                 360                 365

His Phe Gly Gly Ser Trp Lys Asn Asn Gly Lys Gly Glu Ile Lys Pro
    370                 375                 380

Gly Met Glu Gly Tyr Glu Gly Glu Asp Pro Asn Glu Val Glu Glu Leu
385                 390                 395                 400

Arg Lys Asn Asp Val Ser Lys Arg Asp Val Ile Pro Gly Glu Ser Lys
                405                 410                 415

Asp Val Ala Pro Val Lys Lys Leu Ala Lys Arg Cys Ala Tyr Pro Tyr
            420                 425                 430
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Sequence Listing

Thr Pro Tyr
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<210> 4
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<220>
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<400> 4
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<210> 5
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<212> DNA
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<400> 5

Sequence Listing

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<210> 6
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Sequence Listing

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<210> 10
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<400> 10
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<210> 11
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Sequence Listing

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/KR03/01285

A. CLASSIFICATION OF SUBJECT MATTER

IPC7 C12N 15/31

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC7 C12N 15/31

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the intertnational search (name of data base and, where practicable, search terms used)

BLAST, PubMed, Delphion "Hansenula", "mannosyltransferase", "Och"

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO02/00856 A2 (Flanders Interuniversity Institute for Biotechnology) 3 January 2002	1 - 11
X	WO 02/00879A2 (Glycofi Inc.) 3 January 2002	1 - 11
A	NEIMAN A. M. ET AL. "Saccharomyces cerevisiae HOC1, a Suppressor of pkc1, Encodes a Putative Glycosyltransferase", Genetics, March 1997, Vol.145, pages 637-645 (Genetics Society of America)	1 - 11
A	LYNN, M. T. ET AL. "Functional Characterization of the Candida albicans MNT1 Mannosyltransferase Expressed Heterologously in Pichia Pastoris, J. Biol. Chem., June 2000, Vol. 275, No.25, pages 18933-18938 (American Society for Biochemistry and Molecular Biology, Inc.)	1 - 11
A	GELLISSEN, G. ET AL. "Heterologous Protein Production in Methylotrophic Yeast", Appl. Microbiol. Biotechnol., 2000, Vol.54, pages 741-750 (Springer-Verlag) cited in the application	1 - 11

☐ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents,such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
03 SEPTEMBER 2003 (03.09.2003)	04 SEPTEMBER 2003 (04.09.2003)

Name and mailing address of the ISA/KR

Korean Intellectual Property Office
920 Dunsan-dong, Seo-gu, Daejeon 302-701,
Republic of Korea

Facsimile No. 82-42-472-7140

Authorized officer

KWON, Oh Sig

Telephone No. 82-42-481-5773

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.
PCT/KR03/01285

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 02/00856 A2	03/01/2002	US20020188109A1	12/12/2002
		EP1294910A2	26/03/2003
		CA2411968AA	03/01/2002
		AU0177658A5	08/01/2002
WO 02/00879 A2	03/01/2002	US20020137134A1	26/09/2002
		EP1297172A2	02/04/2003
		CA2412701AA	03/01/2002
		AU0176842A5	08/01/2002